Development and specificities of anti-interferon neutralizing antibodies in patients with chronic hepatitis C treated with pegylated interferon-α

C. Scagnolari1, S. Trombetti1, A. Soldà1, M. Milella2, G. B. Gaeta3, G. Angarano4, G. Scotto5, N. Caporaso5, F. Morisco5,6, R. Cozzolongo2, G. Giannelli8, M. Fasano2, T. Santantonio4 and G. Antonelli1

1) Department of Molecular Medicine, Laboratory of Virology, “Sapienza” University of Rome, Rome, 2) Clinic of Infectious Diseases, University of Bari, Bari, 3) Unit of Infectious Diseases, Second University of Naples, Naples, 4) Clinic of Infectious Diseases, University of Foggia, Foggia, 5) Gastroenterology, Department of Clinical and Experimental Medicine, University of Naples “Federico II”, Naples, 6) Department of Food Science, University of Naples “Federico II”, Naples, 7) Division of Gastroenterology I, “S. de Bellis” Hospital—IRCCS, Castellana Grotte and 8) Department of Internal Medicine, Immunology and Infectious Diseases, Section of Internal Medicine; University of Bari, Bari, Italy

Abstract

Only limited data are available on the development of neutralizing antibodies (NAB) in patients with chronic hepatitis C (CHC) treated with pegylated interferon-α (PEG-IFN-α). The aim of this study was to evaluate the immunogenicity of PEG-IFN-α when administered to CHC patients who had or had not previously received standard interferon-α (IFN-α) therapy. In addition, the specificities of NAB, together with the ability of leucocyte (LE) -IFN-α to re-establish therapeutic responsiveness in NAB-positive patients, were evaluated. NAB were assessed using a quantitative, standardized, virus-induced cytopathic effect assay. The seroconversion rate to PEG-IFN-α was higher in patients who had received previous standard IFN-α treatment than in those treated exclusively with PEG-IFN-α. Also, NAB produced during PEG-IFN-α therapy were unable to neutralize LE-IFN-α entirely, even though they can neutralize several IFN-α subtypes. In addition, the results indicate that a change to LE-IFN-α therapy can be associated with restoration of clinical responses in NAB-positive patients who had become resistant after showing an initial response to PEG-IFN-α treatment. This study emphasizes the importance of evaluating NAB development in CHC patients who become resistant to PEG-IFN-α treatment, and suggests management alternatives for patients who develop NAB.

Keywords: Hepatitis C virus, interferon, neutralizing antibodies, seroconversion, sustained virological response

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Introduction

Combination therapy using pegylated-interferon-α (PEG-IFN-α) plus ribavirin (RBV) is currently the standard regimen for patients with chronic hepatitis C (CHC) infection [1]. This makes it possible to achieve a sustained virological response in about 50% of cases of CHC, genotype 1b. Several factors, including specific virus mutations, host factors (such as age, gender, liver fibrosis, lipid metabolism), and single nucleotide polymorphism in, and expression of, some genes coding for proteins involved in antiviral innate immunity, have been reported as being associated with the therapeutic effects of IFN [2–6]. A few studies explaining the failure of IFN-α therapy have also been published in which the development of neutralizing antibodies (NAB) against IFN-α in CHC patients treated with PEG-IFN-α is described [7–9]. However, there have been few reports relating to this issue and, although there is abundant literature on the seroconversion rate during recombinant IFN-α therapy, many issues relating to NAB development in PEG-IFN-α therapy have yet to be addressed [10].

Within the framework of a study aimed at further characterizing the in vivo immunogenicity of PEG-IFN-α preparations, we aimed to assess the immunogenicity of PEG-IFN-α preparations when administered to CHC patients who had or had not received previous therapy with standard recombi-
nant IFN-α products. In addition, the neutralizing specificities of NAB, together with the ability of natural human IFN-α to re-establish therapeutic responsiveness in non-responder patients with CHC who develop anti-IFN NAB while being treated with PEG-IFN-α, have been evaluated.

Materials and Methods

Patients
Analyses of NAB were performed on serum samples collected from 179 patients with CHC who had been treated with PEG-IFN-α 2a (Pegasys®; Hoffmann-LaRoche, Basel, Switzerland) or 2b (PegIntron®; Schering-Plough, Kenilworth, NJ, USA) plus RBV. The patients were divided into two groups, depending on whether or not they had previously received therapy with standard IFN-α preparations. Serum samples were collected at the beginning of therapy and after 6 months, and were stored at −80°C.

In addition, in this study attention focused on five NAB-positive patients with CHC in whom treatment with PEG-IFN-α had failed, and who therefore had to start a new cycle of IFN therapy. To perform an evaluation of NAB specificities, sera were collected from these patients before starting leukocyte (LE) -IFN-α (Alfaferone®; Alfa Wassermann, Bologna, Italy) plus RBV therapy and stored at −80°C. In one patient, serum samples were collected after 1, 2, 3, 4, 5 and 6 months of therapy.

Written informed consent was obtained from each patient and the ethics committees of the participating institutions approved the study.

Detection of NAB to IFN-α
Antibody titres were determined, as described previously, by a neutralization test against 10 IU/mL of IFN-α [11,12]. Different IFN-α preparations were used: IFN-α 2a (Roferon®; Hoffman-La Roche); IFN-α 2b (Intron®; Schering-Plough); LE-IFN-α (Alfaferone®; Alfa Wassermann); Multiﬁeron® (Swedish Orphan Biovitrum, Stockholm, Sweden) and subtypes of IFN-α (PBL Biomedical Laboratories, Piscataway, NJ, USA). Titres were calculated using the Kawade method and expressed as ten-fold reduction units (TRU/mL), that is, the dilution of serum that reduces 10 IU/mL of IFN to 1 IU/mL [13–15].

Statistical methods
Seroconversion rates were compared using the chi-square test. Two-group comparisons of means were done using Student’s t-test. A simple logistic regression, after adjusting for type of PEG-IFN-α administered and genotype of hepatitis C virus (HCV; genotype 1 versus other genotype) was used to evaluate the degree of variables association between treatment-experienced and IFN-α standard-naïve HCV-positive patients. A p-value <0.05 was considered statistically significant. The analysis was performed using SPSS version 13.0 for Windows.

Results

PEG-IFN-α immunogenicity
The rates of NAB production against IFN-α in patients with CHC who had received PEG-IFN-α therapy exclusively were compared with those of patients who had undergone previous courses of therapy with standard IFN-α products before starting PEG-IFN-α therapy. The demographic and clinical characteristics of both groups of patients with CHC are shown in Table 1. Of the patients with CHC treated with PEG-IFN-α who had received no previous IFN-α therapy, none had detectable levels of NAB before therapy, and only one patient developed NAB to IFN-α 6 months after starting PEG-IFN-α therapy. In contrast, NAB were observed in 8% (4/52) of the CHC patients who had received second-line treatment with PEG-IFN-α after previous therapy with standard IFN-α preparations 6 months after beginning PEG-IFN-α therapy. In one of these patients, NAB positivity was recorded before PEG-IFN-α therapy had started. The results shown in Fig. 1 indicate that the incidence of NAB to IFN-α after 6 months of PEG-IFN-α therapy was lower in patients treated exclusively with PEG-IFN-α than in those who had received standard IFN-α therapy before starting PEG-IFN-α treatment (p <0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A n = 52</th>
<th>Group B n = 127</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male (%)</td>
<td>58</td>
<td>74</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>53 ± 10</td>
<td>48 ± 18</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Genotype (%)</td>
<td>2</td>
<td>2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>1</td>
<td>82</td>
<td>45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>40</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Baseline hepatitis C viraemia, log IU/mL (mean ± SD)</td>
<td>6.17 ± 6.54</td>
<td>5.97 ± 5.98</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Baseline alanine aminotransferase, IU/L (mean ± SD)</td>
<td>127 ± 95</td>
<td>113 ± 86</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Type of IFN-α administered (PEG-IFN-α 2a (%)</td>
<td>67</td>
<td>19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PEG-IFN-α 2b (%)</td>
<td>33</td>
<td>81</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*Means and proportions were compared using Student’s t-test and chi-square tests. The analysis was performed using SPSS version 13.0 for Windows.
In addition, when all variables were included simultaneously in a multiple logistic regression analysis, after adjusting for type of PEG-IFN-α administered and HCV genotype (1b versus other), no significant association was found between any clinical and demographic parameters of the two groups of HCV-positive patients. The only exception was the observation that being not naive for IFN treatment was associated with an increased risk of developing neutralizing antibodies (OR 10.09, 95% CI 0.95–107.13) although this increase was not significant at the conventional level (p = 0.055).

Clinical and demographic characteristics of NAB-positive patients are shown in Table 2. It can be seen that almost all NAB-positive patients did not achieve a virologic response to PEG-IFN-α therapy, as documented by measuring levels of HCV RNA, which never dropped to undetectable levels.

However, we observed that a sustained virological response was reached in only 32% of the NAB-negative patients who had undergone previous courses of conventional IFN-α therapy in contrast to the sustained virological response rate of 70% observed in IFN-α standard naive-HCV-positive patients who did not develop NAB during PEG-IFN-α treatment.

NAB specificities

With the aim of investigating the development of NAB in CHC patients further, our attention focused on another group of five patients with CHC in whom treatment with PEG-IFN-α preparations plus RBV had failed after an initial virological response, and who therefore had to start a new cycle of IFN therapy (Table 3). With the exception of patient 3, these patients had not received IFN-α standard therapy previously. Treatment with PEG-IFN-α plus RBV was interrupted after 3 (patient 3), 6 (patients 4 and 5), and 8 (patient 2) months, respectively, when they were found to have developed NAB. In contrast, patient 1 relapsed after the first cycle of PEG-IFN-α 2b plus RBV therapy and NAB were detected in serum collected from this patient after the interruption of a second cycle of antiviral therapy (9 months). All patients started a new cycle of LE-IFN-α plus RBV (800–1200 mg/day) therapy. To perform an evaluation of NAB specificities, sera samples were collected from these patients before starting treatment with LE-IFN-α plus RBV. Interestingly, the levels of NAB produced during PEG-IFN-α therapy varied greatly (coefficient of variation > 100%; Table 3). The ability of NAB developed during PEG-IFN-α therapy to neutralize other preparations of IFN, such as LE-IFN-α, was then evaluated (Table 3). In particular, no neutralizing activity (<10 TRU/mL) against LE-IFN-α was recorded in sera with an NAB titre of ≤107 TRU/mL against IFN-α 2. Otherwise, sera with an NAB titre of ≥293 TRU/mL against IFN-α 2 were to some extent able to neutralize LE-IFN-α, even though a much greater neutralizing activity against IFN-α 2 compared with that against LE-IFN-α was observed. Similar

FIG. 1. Development of anti-interferon (IFN) neutralizing antibody in patients with chronic hepatitis C (CHC). Analysis was performed before (T0) and after 6 months (T6) of pegylated (PEG) -IFN-α plus ribavirin treatment in patients with CHC who had (white bars) or had not (black bars) been treated previously with a conventional IFN-based regimen. p < 0.05 using the chi-square test.

### TABLE 2. Demographic and clinical characteristics of patients with chronic hepatitis C who had developed neutralizing antibodies

<table>
<thead>
<tr>
<th>Pt</th>
<th>Sex</th>
<th>Age (years)</th>
<th>G</th>
<th>HCV RNA (Log IU/mL)</th>
<th>ALT (IU/mL)</th>
<th>Type of PEG-IFN</th>
<th>Previous IFN standard therapy</th>
<th>NAB (TRU/mL)</th>
<th>Type of response</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>F</td>
<td>39</td>
<td>1b</td>
<td>6.64</td>
<td>6.18</td>
<td>6.87</td>
<td>6.73</td>
<td>271</td>
<td>123</td>
</tr>
<tr>
<td>b</td>
<td>M</td>
<td>62</td>
<td>1b</td>
<td>4.26</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>136</td>
<td>30</td>
</tr>
<tr>
<td>c</td>
<td>F</td>
<td>60</td>
<td>1b</td>
<td>5.13</td>
<td>5.02</td>
<td>4.95</td>
<td>5.44</td>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>d</td>
<td>F</td>
<td>65</td>
<td>2</td>
<td>5.36</td>
<td>5.25</td>
<td>Positive</td>
<td>Positive</td>
<td>109</td>
<td>91</td>
</tr>
<tr>
<td>e</td>
<td>F</td>
<td>45</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>40</td>
<td>35</td>
<td>2b</td>
<td>No</td>
</tr>
</tbody>
</table>

Pt, patient; G, genotype; T0, baseline; T3, T6, T12 after 3, 6, 12 months of pegylated interferon (PEG-IFN) therapy; IU, international unit; ALT, alanine aminotransferase; NAB, neutralizing antibodies; TRU, tenfold reduction units; NR, no response; SVR, sustained virological response.
The present study demonstrates, we believe for the first time, that patients with CHC who have received a previous course of standard IFN-α treatment, have a greater probability of

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Specificity of neutralizing antibodies to interferon-α (IFN-α) in sera from selected patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV RNA (Log IU/mL)</td>
<td>Time of neutralizing antibodies against different IFN-α preparations (TRU/mL)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>LE-IFN</td>
</tr>
<tr>
<td>PC²</td>
<td>S</td>
</tr>
<tr>
<td>T0</td>
<td>Daily</td>
</tr>
<tr>
<td>T6</td>
<td>Twice weekly</td>
</tr>
<tr>
<td>T12</td>
<td>Daily</td>
</tr>
</tbody>
</table>

The fate of NAB after changing to LE-IFN-α therapy

A recovery in clinical response after switching to LE-IFN-α plus RBV was observed in patients 1, 2, and 5, independent of the presence in their sera of NAB against natural IFN-α preparations (Table 3). In contrast, patient 4, who had a measurable NAB titre against natural IFN-α preparations, did not respond to LE-IFN-α therapy.

In addition, we were able to evaluate the changes in NAB specificities to IFN-α during LE-IFN-α treatment for patient 2. The results are shown in Fig. 2. It can be seen that over 6 months of LE-IFN-α therapy, no neutralizing activity against natural IFN-α preparations was observed, nor were changes seen in NAB specificities to almost all IFN-α subtypes, with the exception of the development of NAB against the IFN-α 10 subtype. The NAB titre against some IFN-α subtypes also showed a weak increase during LE-IFN-α therapy.

Discussion

The present study demonstrates, we believe for the first time, that patients with CHC who have received a previous course of standard IFN-α treatment, have a greater probability of
developing NAB after administration of PEG-IFN-α than patients being treated for the first time with this preparation.

This important finding suggests that the long-term administration of different types of chemical products with the same IFN-α subtype could induce an immunological response even towards PEG-IFN-α preparations, which have been shown to possess lower immunogenicity compared with those reported for the standard preparations of IFN-α [16–18]. The seroconversion rate to PEG-IFN-α observed in our study is also lower than those reported recently [9]. However, it should be noted that Halfon et al. [9] measured NAB levels in sera collected from patients with CHC with an ELISA test, which detects antibodies that bind several different antigenic epitopes of the IFN-α molecule, some of which are not involved in the activation of type I IFN receptors and are recognized only by binding but not neutralizing antibodies. In contrast, in our own analysis, we used a universally employed, quantitative, standardized virus-induced cytopathic-effect assay based on IFN induction of antiviral resistance, which is considered to represent the reference standard for measurement of NAB [14].

This is the first study in which NAB specificities produced during PEG-IFN-α therapy have been analysed. It is of interest that only sera with high NAB titres against the IFN-α 2 subtype had the ability to neutralize, even though to a lower and different extent, most of the IFN-α subtypes, and, consequently, natural IFN-α preparations. In contrast, only a few subtypes of IFN-α were neutralized by those sera with low titres of NAB against the IFN-α 2 subtype. The ability of NAB that developed against IFN-α 2 to recognize other closely related type I IFN-α subtypes has also been reported in patients with CHC, autoimmune diseases or tumours treated with conventional IFN-α preparations [11,19–22].

Specifically, our results show that the IFN-α 6 subtype was recognized by all sera positive for NAB to IFN-α 2, whereas IFN-α 1 subtypes were only marginally neutralized. In agreement with this, Nolte et al. [23] showed that NAB that developed in patients suffering from leukaemia during IFN-α 2 treatment, neutralized IFN-α 6 but not (or only marginally) IFN-α 1. The different IFN-α subtype specificities of NAB produced during PEG-IFN-α therapy may be explained by the observation that some amino acids within residues 17–65 of the N-terminal functional domain of the IFN-α 2 subtype, recognized by neutralizing therapy-induced NAB, are identical in IFN-α 2 and IFN-α 6 subtypes but different in IFN-α 1 [23].

As far as the clinical impact of NAB developed during PEG-IFN-α therapy is concerned, our results confirm that the development of NAB may be one of the host factors associated with the lack of clinical response to IFN-α therapy [7,8,11,18,24–26]. This seems particularly evident for the group of HCV-positive patients who became resistant to PEG-IFN-α treatment concomitantly with the development of anti IFN neutralizing antibodies and who therefore had to start a new cycle of LE-IFN-α therapy. However, a lack of virological response after 3 months of PEG-IFN-α therapy was observed in most IFN-α standard treatment-experienced NAB-positive patients. In addition, the rate of sustained virological response was also low in NAB-negative patients who underwent a second course of IFN therapy with a PEG-IFN-α.
**α** preparation. This finding seems to be expected considering that most patients who previously had no response to standard IFN therapy independently of their NAB status were infected with HCV genotype 1, which is known to show a worse treatment response compared with the other HCV genotypes.

In addition, one patient, who had received a previous conventional IFN-**α** preparation, showed a positive clinical response to PEG-IFN-**α** therapy, despite the presence of NAB. Achievement of a sustained virological response despite the presence of NAB could be explained in this case by hypothesizing that this patient developed NAB after the decline in HCV-RNA to undetectable levels observed in the first few months of therapy. This finding is not surprising and does not seem to be confined solely to HCV-positive patients. Indeed, it has been reported that NAB cannot affect the clinical efficacy of IFN-**β** treatment in certain patients with multiple sclerosis who clinically responded to IFN before the occurrence of seroconversion [27].

These observations indirectly emphasize the complexity of the phenomenon analysed and the difficulty in interpreting the data on the evaluation of the influence of NAB development in the outcome of PEG-IFN-**α** therapy in patients with CHC.

Furthermore, and more importantly from a practical viewpoint, our results indicate that a second course of treatment with the natural IFN preparation may be useful in most anti-interferon NAB-positive patients who fail to respond to PEG-IFN-**α** plus RBV therapy as previously described [8,28,29].

A recovery in clinical response to natural IFN-**α** was also observed in one patient who had NAB against virtually all IFN-**α** subtypes before starting LE-IFN-**α** plus RBV therapy. This finding may suggest that some IFN-**α** subtypes present in the mixture of the natural IFN-**α** preparation, which were neutralized to a low extent, might still exert their biological activity in patients with CHC as reported by others [8,10,11]. Nonetheless, we consider that the observation that natural IFN-**α** preparation could induce a widening of NAB specificities to IFN-**α** subtypes should be taken into consideration in the clinical management of patients who, as non-responders to PEG-IFN-**α** therapy, have developed NAB and who can be successfully re-treated with different IFN-**α** preparations or with new anti-HCV drugs.

In conclusion, our data show that PEG-IFN-**α** preparations possess an immunogenic potential when administered to CHC patients who have previously received conventional IFN-**α** therapy. Our data also indicate that NAB that developed in patients with CHC during PEG-IFN therapy are not able to neutralize natural IFN-**α** entirely, even though they possess a broad specificity of neutralizing activity. In addition, our data suggest that the presence of NAB to individual IFN-**α** species will not significantly diminish the clinical efficacy of natural IFN-**α** preparations. Given the impact of NAB on clinical response to PEG-IFN-**α** therapy, our results taken as a whole underline the importance of evaluating NAB production, in patients who have become resistant after showing an initial response to PEG-IFN-**α** treatment and have undergone previous therapy with standard IFN-**α**, to provide therapeutic alternatives for managing NAB-positive patients.

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**Transparency Declaration**

The authors declare no conflict of interest.

**References**


